JOURNAL OF CLINICAL MICROBIOLOGY, July 2002, p. 2392–2397 Vol. 40, No. 7
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.7.2392–2397.2002

Detection of Methicillin-Resistant *Staphylococcus aureus* and Simultaneous Confirmation by Automated Nucleic Acid Extraction and Real-Time PCR

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Received 8 February 2002/Returned for modification 20 March 2002/Accepted 10 April 2002

A molecular assay for the simultaneous detection of a *Staphylococcus aureus*-specific gene and the *mecA* gene, responsible for the resistance to methicillin in staphylococci, was evaluated. The assay included an automated DNA extraction protocol conducted with a MagNA Pure instrument and real-time PCR conducted with a LightCycler instrument. The performance and robustness of the assay were evaluated for a suspension of methicillin-resistant *S. aureus* (MRSA) strain with a turbidity equivalent to a McFarland standard of 0.5, which was found to be the ideal working concentration. The specificity of the new molecular assay was tested with a panel of 30 gram-negative and gram-positive bacterial strains other than MRSA. No cross-reactivity was observed. In a clinical study, 109 isolates of MRSA were investigated. All clinical MRSA isolates gave positive results for the *S. aureus*-specific genomic target, and all but one were positive for the *mecA* gene. In conclusion, the new molecular assay was found to be quick, robust, and laborsaving, and it proved to be suitable for a routine molecular diagnostic laboratory.

Staphylococcus aureus has been known to be a major pathogen causing a wide spectrum of clinical manifestations, such as wound infections, pneumonia, septicemia, and endocarditis, with beta-lactam antibiotics being the drugs of choice for therapy. Since the introduction of methicillin into clinical use in 1961, the occurrence of methicillin-resistant *S. aureus* (MRSA) has steadily increased and nosocomial infections caused by such isolates have become a serious problem worldwide (2, 14).

The differentiation of MRSA strains from other strains of *S. aureus* has important implications for the treatment and management of patients with *S. aureus* infections, and glycopeptides are the drugs of choice for infections caused by MRSA strains. Furthermore, evidence of MRSA requires extensive hygienic precautions to limit the spread of such strains (5, 34).

In the clinical laboratory, *S. aureus* is identified by growth characteristics and by the subsequent detection of catalase and coagulase activities. Conventional susceptibility testing of *S. aureus* reliably detects resistance to methicillin or oxacillin if agar dilution tests, disk diffusion tests, or agar screening methods are used according to the standards of the National Committee of Clinical Laboratoy Standards (NCCLS) (16, 17). Standard susceptibility tests, however, are time-consuming. Because the phenotypic expression of methicillin resistance in vitro is heterogeneous and sometimes difficult to induce, false-negative results may be observed (20, 27). Furthermore, *S. aureus* strains may show a false-negative or noninterpretable result when commercially available kits for coagulase testing (8, 21, 29, 33, 35) are used.

The main mechanism of methicillin resistance is induced by the presence of an additional low-affinity penicillin-binding protein, PBP 2a (encoded by the *mecA* gene), or, in rare cases, induced by the hyperproduction of β-lactamase (6, 10, 32). Detection of the *mecA* gene by PCR has been described as a rapid method for the identification of MRSA (1, 4, 11, 13, 15, 23, 24, 28, 36).

In the present study, a molecular assay for the rapid identification of MRSA was established and evaluated. The new assay targeted both the *S. aureus* gene and the *mecA* gene within a single PCR and was based on automated DNA isolation and real-time PCR.

MATERIALS AND METHODS

Study design. A molecular assay for the detection of MRSA based on automated DNA extraction and real-time PCR was established. The new assay was based on automated DNA extraction with a MagNA Pure LC instrument (Roche Molecular Biochemicals, Mannheim, Germany) and real-time PCR with a LightCycler instrument (Roche Diagnostics, Mannheim, Germany).

In the first step, a suspension of the MRSA strain NCTC 10442 (National Collection of Type Cultures and Pathogenic Fungi, Colindale, United Kingdom) with a turbidity equivalent to a McFarland standard of 0.5 (corresponding to 1.5×10^8 CFU per ml) was prepared. The molecular assay based on the automated DNA extraction protocol and real-time PCR on the LightCycler instrument was evaluated. The experiments were repeated five times on different days.

In the second step, the specificity of the new molecular assay was determined with a panel of 30 gram-negative and gram-positive bacterial strains other than MRSA. These included the *Escherichia coli* standard strain ATCC 25922 (American Type Culture Collection, Manassas, Va.), the *Pseudomonas aeruginosa* standard strain ATCC 27853, the *Enterococcus faecalis* standard strain ATCC 29212, and 21 strains of coagulase-negative *Staphylococcus* spp. (*S. saprophyticus*, *S. epidermidis*, *S. warneri*, and *S. haemolyticus*) which had been identified in routine diagnostic laboratories. Furthermore, three oxacillin-susceptible *S. aureus* standard strains (ATCC 29213, ATCC 25923, and NCTC 8325) and three oxacillin-susceptible *S. aureus* strains which had been isolated in a routine diagnostic laboratory were tested.

In the third step, 109 clinical MRSA isolates were tested. Samples were derived from wounds (49 samples), the respiratory tract (41 samples), and the urinary tract (19 samples). *S. aureus* strains were identified by their characteristic growth morphologies, Gram stain characteristics, reaction to catalase, coagulase production as detected with the Staphaurex Plus system (Murex Biotech Ltd, Dartford, United Kingdom), the results of the ID32 Staph Apitest (Biomerieux,

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TABLE 1. Oligonucleotide primers and LightCycler hybridization probes used in the PCR assay

Oligonucleotide	Sequence[a]	Target gene	Nucleotide positions	GenBank accession no.	Source
Sa442-F	GTCGGGTACACGATATTCTTCACG	*Sa442*	12–34	AF033191	This study
Sa442-RS	CTCGTATGACCAGCTTCGGT	*Sa442*	189–168	AF033191	This study
Sa442-HP-1	TACTGAAATCTCATTACGTTGCATCGGAA-FAM	*Sa442*	95–123	AF033191	Reischl et al. (19)
Sa442-HP-2	Red 705-ATTGTGTTCTGTATGTAAAAGCCGTCTTG-Ph	*Sa442*	126–154	AF033191	Reischl et al. (19)
Mec-S	CTAGGTGTGGTGAAGATATACCA	*mecA*	1596–1619	X52592	This study
Mec-A	TGAGGTGCGTTAATATTGCCA	*mecA*	1783–1763	X52592	This study
Mec-HP-1	CAGGTTACGGACAAGGTGAAATACTGATT-FAM	*mecA*	1690–1718	X52592	Reischl et al. (19)
Mec-HP-2	Red 640-ACCCAGTACAGATCCTTTCAATCTATAGCG-Ph	*mecA*	1720–1739	X52592	Reischl et al. (19)

[a] FAM, fluorescein; Red 705, LightCycler Red 705 phosphoramidite; Ph, 3′-phosphate; Red 640, LightCycler Red 640 *N*-hydroxysuccinimide ester.

Marcy l'Etoile, France), and identification with an ID-GPC card (bioMerieux Vitek, Inc., Hazelwood, Mo.) on a VITEK-2 instrument (bioMerieux Vitek, Inc.). Resistance to oxacillin was determined to be indicated by a MIC of ≥4 μg/ml and was tested with the oxacillin Etest (AB Biodisk, Solna, Sweden), according to the guidelines of the NCCLS, and with an AST-P523 card (bioMerieux Vitek, Inc.) on the VITEK-2 instrument. After identification, the strains were stored at −70°C. For molecular testing, clinical specimens were thawed and recultured on blood agar overnight at 37°C. Portions of individual bacterial colonies were suspended in 200 μl of phosphate-buffered saline buffer.

DNA extraction. The isolation of bacterial DNA was done on a MagNA Pure LC, a benchtop instrument that can extract 32 samples in parallel (12). For DNA extraction, we used a MagNA Pure LC DNA Isolation Kit III (standard protocol; Roche Molecular Biochemicals). The sample volume was 200 μl (a suspension with a turbidity equivalent to a McFarland standard of 0.5); the elution volume was 100 μl. After the completion of the DNA extraction, the MagNA Pure LC cooling block, including the sample carousel with an adequate number of LightCycler capillaries, and the reaction vessels, including the master mix, were placed into the postelution area. After the start of the postelution protocol, which had been programmed prior to the start of the first run, the MagNA Pure LC automatically pipetted 18μl of the master mix and 2 μl of the processed sample into each of the LightCycler capillaries.

Primers and probes. Oligonucleotide primers and fluorescence-labeled hybridization probes were designed for amplification and sequence-specific detection of both a 188-bp fragment within the *mecA* gene and a 178-bp fragment within the *S. aureus*-specific *Sa442* gene. The primers and probes were obtained from TIB MolBiol (Berlin, Germany). The nucleotide sequences and positions are listed in Table 1.

The master mixture contained 2 μl of a 10× mixture of LightCycler FastStart DNA master hybridization probes (Roche Diagnostics), 5 mM $MgCl_2$ (final concentration), a 1 μM final concentration of *mecA* primers, a 0.075 μM final concentration of *S. aureus*-specific primers, and a 0.2 μM final concentration of hybridization probes.

Real-time PCR. After the completion of the postelution protocol, the LightCycler capillaries were sealed. Then, the sample carousel with the capillaries was centrifuged in the LightCycler carousel centrifuge and placed into the LightCycler instrument. The cycling protocol consisted of one cycle of 10 min at 95°C followed by 50 cycles consisting of denaturation for 10 s at 97°C, annealing for 10 s at 50°C, and elongation for 15 s at 72°C. After the final cycle, the capillaries were cooled for 2 min at 40°C. Fluorescence curves were analyzed with the LightCycler software (version 3.5.3). Automated calculation of crossing points was done by the second-derivative maximum method. The fluorescence of each capillary was measured at wavelengths of 640 and 705 nm (dual-color option). We selected cycles from 0 to 50 and channel F2/F1 for the *mecA* gene and channel F3/F1 for the *S. aureus*-specific gene. Each run contained the MRSA standard strain *S. aureus* NCTC 10442 and two negative controls (blank reagent and water). Each result was confirmed by the specific peak in the corresponding melting curve.

RESULTS

When a suspension of an MRSA strain with a turbidity equivalent to a McFarland standard of 0.5 was repeatedly tested by a molecular assay based on the automated DNA extraction protocol and real-time PCR on a LightCycler instrument, the crossing points of both targets were always found to be within one cycle. With regard to melting temperatures, no significant differences were observed.

Specificity testing gave negative results for both the *mecA* gene and the *S. aureus*-specific gene when we tested the *E. coli*, *P. aeruginosa*, and *Enterococcus faecalis* standard strains. Of all coagulase-negative *Staphylococcus* strains, the methicillin-resistant strains showed positive results for the *mecA* gene but negative results for the *S. aureus*-specific marker. All oxacillin-susceptible *S. aureus* strains gave positive results for the *S. aureus*-specific gene but negative results for the *mecA* gene.

All clinical MRSA isolates showed positive results for the *S. aureus*-specific gene. Of 109 MRSA strains, 108 gave positive results for the *mecA* gene; one of the tested MRSA strains gave a negative result for the *mecA* gene. The melting point curves for each of the targets showed identical product peaks. The results for four clinical samples are shown in Fig. 1.

The whole molecular assay was completed within 4 h. The automated DNA extraction with the MagNA Pure LC took 115 min for the extraction of 32 samples. This included a 15-min setup of the MagNA Pure LC. The time required for the postelution protocol was 30 min. After centrifugation, the LC-PCR took another 55 min. No contamination was observed at any time during the study.

DISCUSSION

Since the introduction of semisynthetic penicillins, such as methicillin and oxacillin, for the therapy of infections caused by *S. aureus*, the occurrence of *S. aureus* strains resistant to methicillin has steadily increased and MRSA strains have become major nosocomial pathogens (18, 30). Infections with MRSA strains require treatment with glycopeptide antibiotics, which can be nephro- and ototoxic. Additionally, a diagnosis of MRSA infection has important implications for the management of patients since an extensive set of hygienic precautions must be taken to limit the spread of MRSA (3).

Therefore, a rapid and reliable diagnosis of infection by MRSA is of major importance. Although *S. aureus* is relatively easy to cultivate, conventional identification methods may yield false-positive or false-negative results (9, 31). Standard susceptibility tests are time-consuming. The correct identification of *S. aureus* and the detection of the *mecA* gene based on molecular methods have evolved as the method of choice for definitive identification. Earlier studies, however, involved more or less complicated manual DNA extraction protocols, followed by single or multiplex PCR with detection of ampli-

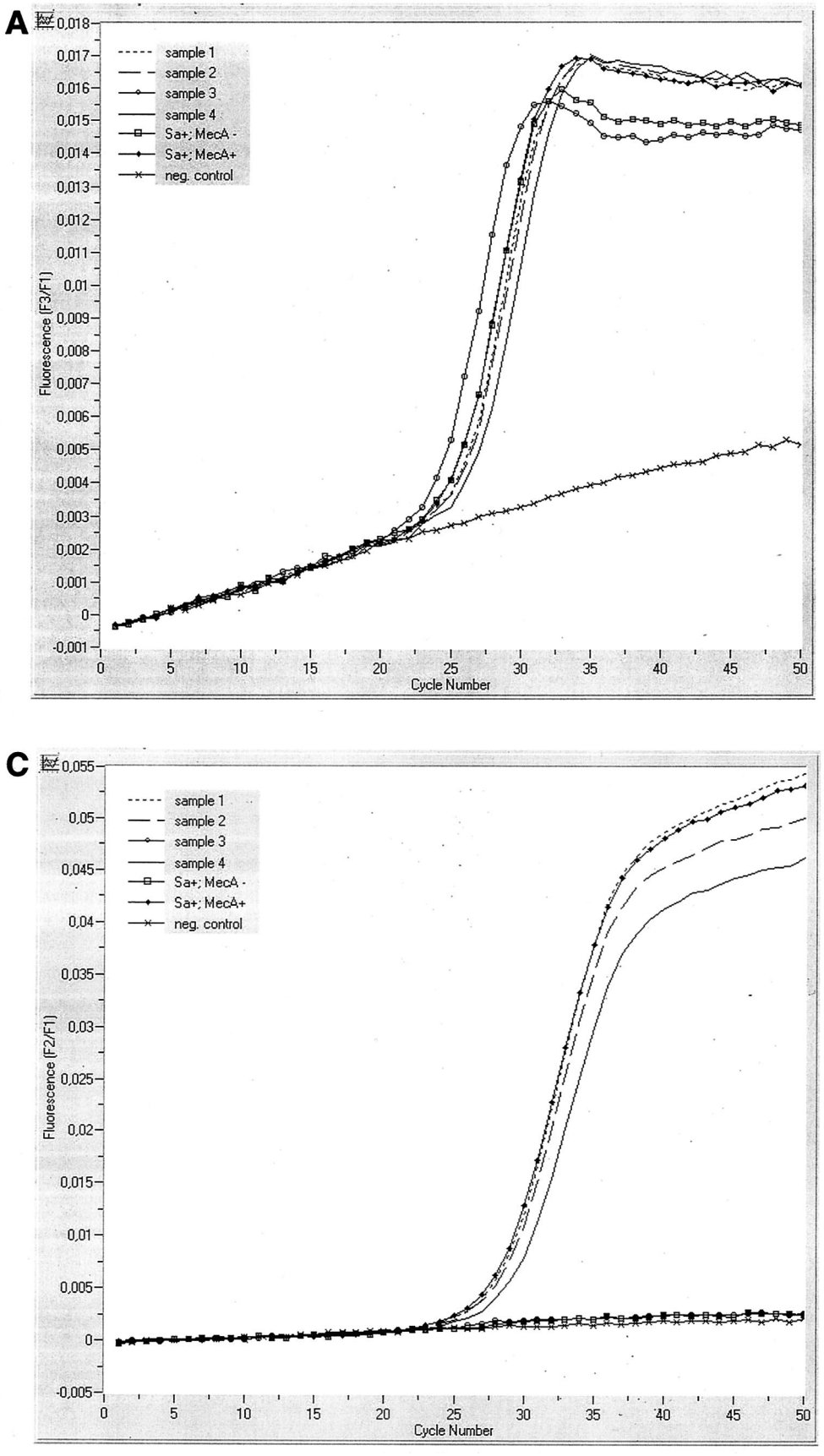
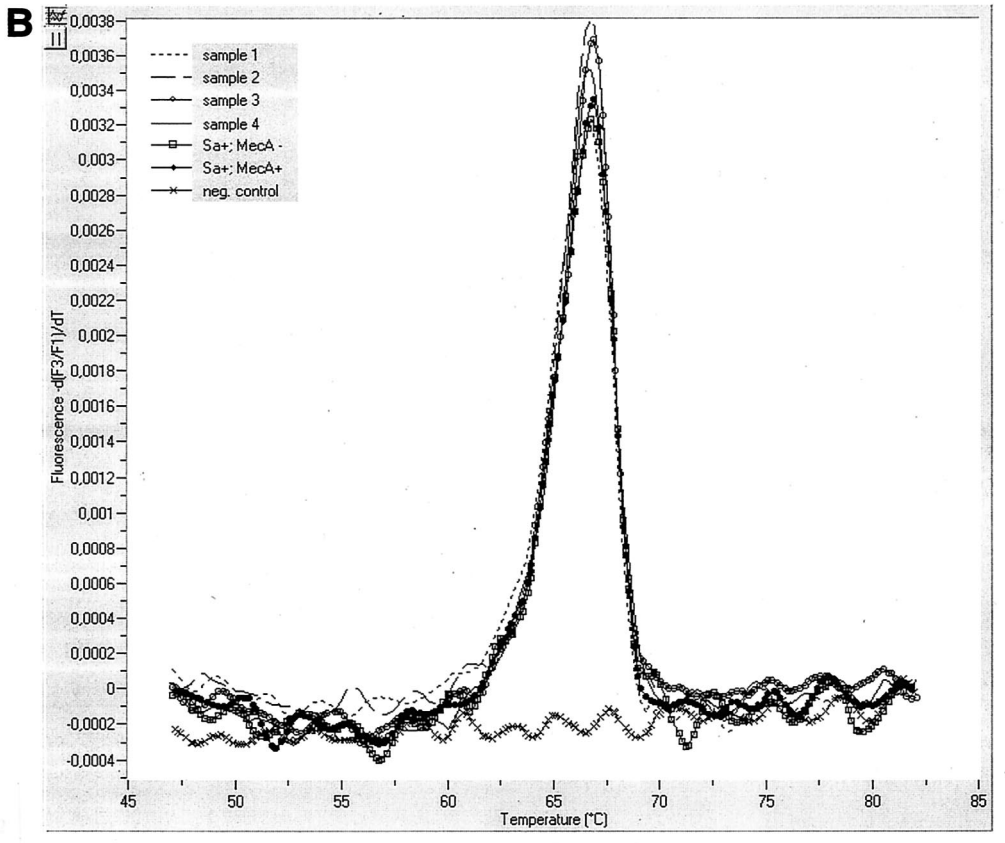
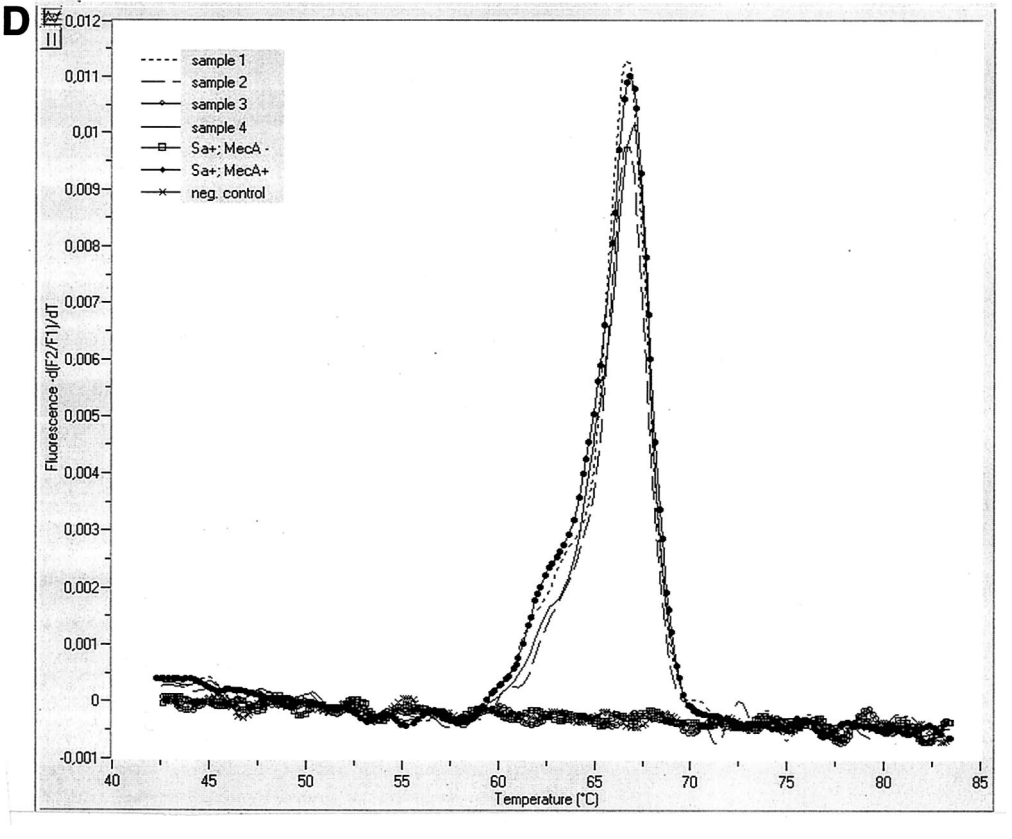


FIG. 1. (A) Fluorescence versus cycle number plots (*S. aureus*-specific genomic fragment *Sa442*) for clinical samples; (B) melting point curves for the samples in panel A showing identical product peaks; (C) fluorescence versus cycle number plots (*mecA* gene) of clinical samples; and (D) melting point curves for the samples in panel C showing identical product peaks.


B
sample 1
sample 2
sample 3
sample 4
Sa+; MecA -
Sa+; MecA+
neg. control
Fluorescence -d[F3/F1)/dT
Temperature (°C)


D
sample 1
sample 2
sample 3
sample 4
Sa+; MecA -
Sa+; MecA+
neg. control
Fluorescence -d[F2/F1)/dT
Temperature (°C)

fication products on agarose gels (1, 4, 11, 13, 15, 23, 24, 28, 36). Those assays, however, were time-consuming, prone to contamination, and not suitable for routine diagnostic laboratories because of the lack of a hybridization technique.

Recently, a real-time PCR technique for the detection of MRSA with two separate PCRs based on a manual DNA extraction protocol was described (22). In that study, however, no internal control was employed. Reischl et al. (19) described a manual DNA extraction protocol followed by multiplex real-time PCR for the simultaneous detection of the *mecA* gene and an *S. aureus*-specific gene which served as an internal control. In comparison to the conditions used in that study, primer compositions and concentrations had to be changed in our study to balance PCR efficiencies for both of the targets by optimization of primer concentrations and product lengths. Because variations in lot-to-lot primer concentrations may exist, it is advisable to adjust the concentrations for each primer lot prior to its first use in routine diagnostics. Following the optimization of the primer concentrations, identical melting point curves for the targets could be shown in this study.

In spite of the growing consensus for the use of molecular methods, they are not yet available in the majority of routine diagnostic laboratories because of their elevated technical requirements. In the present study, a new molecular assay that targets both an *S. aureus*-specific gene and the *mecA* gene within a single PCR was established and evaluated. This assay includes an automated DNA extraction protocol on a MagNA Pure instrument and real-time PCR on a LightCycler instrument. Of 109 clinical MRSA strains, 108 could be detected distinctly by the new molecular assay. One isolate showed a negative result for the *mecA* gene. The reason for this might be one of the rare other mechanisms of methicillin resistance (7, 25, 26).

In conclusion, the new molecular assay was found to be rapid and robust. Because it is a largely automated assay, less hands-on work is needed and it can be incorporated into the workflow of a routine diagnostic laboratory.

ACKNOWLEDGMENT

We gratefully thank Olfert Landt for providing the modified primers and for stimulating discussions.

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